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ENDOCRINE DISRUPTING POTENTIAL IN FRESHWATER ECOSYSTEMS NEAR AGRICULTURAL AREAS ON PRINCE EDWARD ISLAND

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**ENDOCRINE DISRUPTING POTENTIAL IN FRESHWATER
ECOSYSTEMS NEAR AGRICULTURAL AREAS
ON PRINCE EDWARD ISLAND**

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ABSTRACT

There has been increased concern over the release of potential endocrine disrupting compounds into the environment. Environment Canada recently identified intensive agricultural activity as a priority area for exploring possible endocrine disrupting effects. A particularly suitable area in which to assess these effects is Prince Edward Island, where approximately 50% of the land is agricultural and pesticide use is some of the most intensive in Canada. One component of the study measured the effects on endocrine function in caged rainbow trout (*Oncorhynchus mykiss*) and wild brook trout (*Salvelinus fontinalis*). Rainbow trout were caged in Prince Edward Island streams in regions of low, medium, and high agricultural risk based on qualitative assessments of sites according to intensity of agriculture, topography and riparian conditions. Exposure periods were for 21 or 42 days, from mid-July to mid-September that encompassed the most active pesticide spray period. Both immature male and immature female fish (approx. 18-19 months old at the time of deployment) were analyzed for concentrations of the steroid hormone testosterone in blood. Although there were some sites with significantly increased and decreased concentrations of testosterone, there was no clear relationship between testosterone concentrations and risk of exposure to agricultural endocrine disruptors. Attempts were made to quantify the vitellogenin levels in the blood samples of the immature male fish however, sample handling and analytical problems may have contributed to unreliable data. Histological analyses of gonadal tissues in wild male brook trout collected in May 1998 from areas of high intensity potato growing areas showed no evidence of ova development in the testes (testis-ova). Blood from wild brook trout was also collected in September 1998 from regions of medium to high agricultural intensity and also from two rivers within forested areas of eastern PEI as reference sites. The plasma concentrations of 17 β -estradiol and 11-ketotestosterone were used for determining gender however the concentrations of these two steroids as well as testosterone did not indicate any effects associated with agricultural activity.

Another component of the study involved exposing Japanese medaka (*Oryzias latipes*) embryos to sediments collected from each of the caging sites. Fertilized eggs were exposed to sediments in the laboratory from Day 1 to Day 12 of development. Mortality and failure to inflate a swim bladder were greater in eggs and larvae exposed to sediments from high intensity sites compared to low intensity sites. Larvae exposed to sediment from high risk sites also took longer to hatch than those exposed to low risk sediment.

The results from the steroid hormone analyses and the medaka embryotoxicity assay indicate that there may be some effects due to agricultural activities. Whether these effects are a result of endocrine disruption requires further research.

RÉSUMÉ

L'inquiétude entourant le rejet des composés perturbateurs du système endocrinien dans l'environnement grandit sans cesse. Environnement Canada a récemment fait de l'activité agricole sa cible première pour étudier les effets possibles des perturbateurs endocriniens. L'Île-du-Prince-Édouard est un endroit idéal pour évaluer ces effets, car environ 50 % de ses terres sont à vocation agricole. De plus, l'utilisation des pesticides dans cette province est parmi les plus intensives au Canada.

Une partie de l'étude consistait à mesurer les effets des pesticides sur le système endocrinien de la truite arc-en-ciel (*Oncorhynchus mykiss*) en cage et de l'omble de fontaine (*Salvelinus fontinalis*) sauvage. On a mis en cage des truites arc-en-ciel dans des cours d'eau de l'île situés dans des régions où le risque lié à l'activité agricole était, selon des évaluations qualitatives, faible, moyen ou élevé; ces évaluations se fondaient sur l'intensité de l'activité agricole, la topographie et les conditions riveraines. Les expositions ont duré 21 ou 42 jours, de la mi-juillet à la mi-septembre, c'est-à-dire pendant la période d'épandage de pesticides la plus active. On a mesuré dans le sang des poissons immatures mâles et femelles (âgés d'environ 18-19 mois au début de l'étude) les concentrations de testostérone, une hormone stéroïde. Même si les concentrations de testostérone à certains sites ont augmenté ou diminué de façon importante, on n'a pas trouvé de rapport évident entre les concentrations de testostérone et le risque d'exposition aux perturbateurs endocriniens provenant des activités agricoles. On a tenté de quantifier les niveaux de vitellogénine dans les échantillons de sang des poissons mâles immatures; toutefois, des problèmes de manipulation des échantillons et d'analyse font que les données sont peu fiables. L'analyse histologique des tissus gonadiques d'ombles de fontaine mâles sauvages prélevés en mai 1998 dans des zones de culture intensive de la pomme de terre n'ont montré aucun signe de production d'ovules par les testicules.

En septembre 1998, on a également prélevé du sang d'ombles de fontaine sauvages vivant dans les régions où l'activité agricole est moyenne à élevée, et dans deux rivières témoins situées dans des régions boisées de l'est de l'Île-du-Prince-Édouard. Les concentrations plasmatiques du 17 - estradiol et de la 11-cétotestostérone ont été utilisées pour déterminer le sexe des poissons libérés (le sexage n'a pu être fait visuellement). Les concentrations de ces deux stéroïdes et de la testostérone n'ont cependant révélé aucun effet associé à l'activité agricole.

Une autre partie de l'étude touchait l'exposition des embryons d'un poisson japonais, le médaka (*Oryzias latipes*), aux sédiments recueillis à chaque site de parcage de la truite arc-en-ciel. En laboratoire, on a exposé les œufs fécondés aux sédiments du jour 1 au jour 12 de leur croissance. La mortalité et l'absence de gonflement de la vessie natatoire étaient plus marquées chez les larves et les œufs exposés aux sédiments provenant de sites où l'activité agricole est élevée que chez ceux exposés aux sédiments des sites où l'activité agricole est faible. Les œufs exposés aux sédiments des sites d'activité agricole élevée prenaient également plus de temps à éclore que ceux exposés aux sédiments des sites d'activité agricole faible.

Les résultats des analyses des hormones stéroïdes et de l'essai sur l'embryotoxicité chez le médaka indiquent que les activités agricoles exercent peut-être certains effets. Pour déterminer si ces effets résultent d'une perturbation endocrinienne, il faudrait effectuer une recherche plus approfondie.

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1.0 Introduction

In the past 50 years, there has been a dramatic increase in the numbers and amounts of anthropogenic chemicals produced and released into the environment (Colborn *et al.* 1993). It has become apparent that some of these chemicals may disrupt the endocrine system of fish, wildlife, and possibly humans (Colborn *et al.* 1993). The potential impacts of endocrine disrupting chemicals (EDCs) on the immune, nervous, and reproductive systems of organisms has made this a priority issue for governments and researchers worldwide. Environment Canada has been aware that the issue of chemically induced endocrine disruption is not a new one - research has been ongoing in this field for the past three decades (Freeman and Idler 1975; Spies and Rice 1988; Jobling *et al.* 1998). Recently, public attention has increased due to some high profile studies suggesting possible endocrine disrupting chemicals in the environment related to pesticide use (Fairchild *et al.* 1999). The issue has created great debate among researchers, as some feel that endocrine disruption may have serious consequences on humans as well as those already demonstrated in fish and wildlife populations (Colborn *et al.* 1996).

Environment Canada held a workshop in the fall of 1997 to establish priorities for research efforts in the study of endocrine disruptors (Environment Canada (EC) 1997). Two key areas were identified as having the potential for endocrine disruption: (a) sites with intensive agriculture (pesticides and animal waste inputs), and (b) sites receiving urban effluent (especially sewage). The workshop also placed a high priority on the development and validation of new tools to detect the effects of endocrine disruption in the Canadian environment (Environment Canada (EC) 1997).

1.1 Pesticide Use on PEI

The agricultural practices on Prince Edward Island are among the most intensive of any province in Canada (PEI Roundtable 1997) and the potato crop represents the largest cash crop on the island (P. Boswall, pers. comm. 1998). Fungicide usage has increased over the past three years due to weather conditions that are ideal for the development and spread of

late blight and the new A2 strain of late blight (P. Boswall, pers. comm. 1998). When conditions appear favourable for blight development, farmers take prophylactic measures resulting in a regular application schedule of periods between 5-10 days. It has been estimated that an individual potato crop on PEI may receive up to 19 applications of various pesticides in a single growing year.

1.2 Project Objectives

The aim of this study was to determine the potential for endocrine disruption in regions influenced by intense agricultural activity. As this was a pilot study, the goal was to identify effects from agricultural practices in general rather than determine the specific causative agents. The detection of vitellogenin, a female-specific protein, in male fish and the levels of circulating reproductive hormones in fish have been used previously by Environment Canada researchers to detect potential endocrine disruption (i.e. lab exposures and industrial effluent monitoring) (Munkittrick *et al.* 1992a, 1992b, 1994; McMaster *et al.* 1992, 1994; Sherry *et al.* 1998). The Japanese medaka assay is an embryolarval fish assay that is used to assess acute endpoints related to the exposure of the fish to chemicals, complex mixtures or environmental samples. In this project, we attempted to 1) quantify vitellogenin concentrations in the plasma of caged rainbow trout, 2) examine changes in steroid concentrations in the plasma samples of both caged and wild fish and 3) assess early development in medaka exposed to sediment collected from regions of different agricultural intensity.

2.0 METHODS AND MATERIALS

2.1 Study Sites

For rainbow trout caging, sites were selected based on reconnaissance of prospective rivers in order to locate suitable caging locations on each of selected rivers. All sites were required to have (i) adequate road access, (ii) one or more areas along the river deep enough to submerge a fish cage, and (iii) with adequate water flow. In total, eighteen sites on eight rivers were selected.

Figure 1 indicates the study areas and identifies the caging sites. The study sites were ranked based on the potential for agricultural contamination. The rankings were determined largely from visual observations with the higher potential where agricultural fields surrounded, or were adjacent to, or immediately upstream of the caging site and the lower potential assigned to those sites where there were no visible fields adjacent to or upstream of the sites or where the sites were surrounded by mainly forested land (M.Gray pers. obs.). These visual observations were combined with summary information regarding hydrogeological aspects of the land surrounding the sites such as slope of the surrounding land, size of drainage basin, width of the stream, etc. (D. McCall, pers. comm. 1998). The sites were separated into three classes: low, medium and high potential risk. The lowest risk group consisted of sites 1-6; the medium risk group consisted of sites 8, 9, 10, 13, 14, and 18; and the highest risk group consisted of sites 7, 11, 12, 15, 16, and 17. Table 1 summarizes the site numbers with their respective river names and their classification in the three risk groups.

For the wild fish sampling, brook trout were collected in May 1998 from sites classified as medium to high risk within western and central PEI (Wilmot R., Dunk R., Indian R., Bradshaw R.). In September 1998, fish were collected from the same sites as well as from two reference rivers within forested areas of eastern PEI (Bear R., Hay R.).

Figure 1. Caged Fish Sites on Prince Edward Island

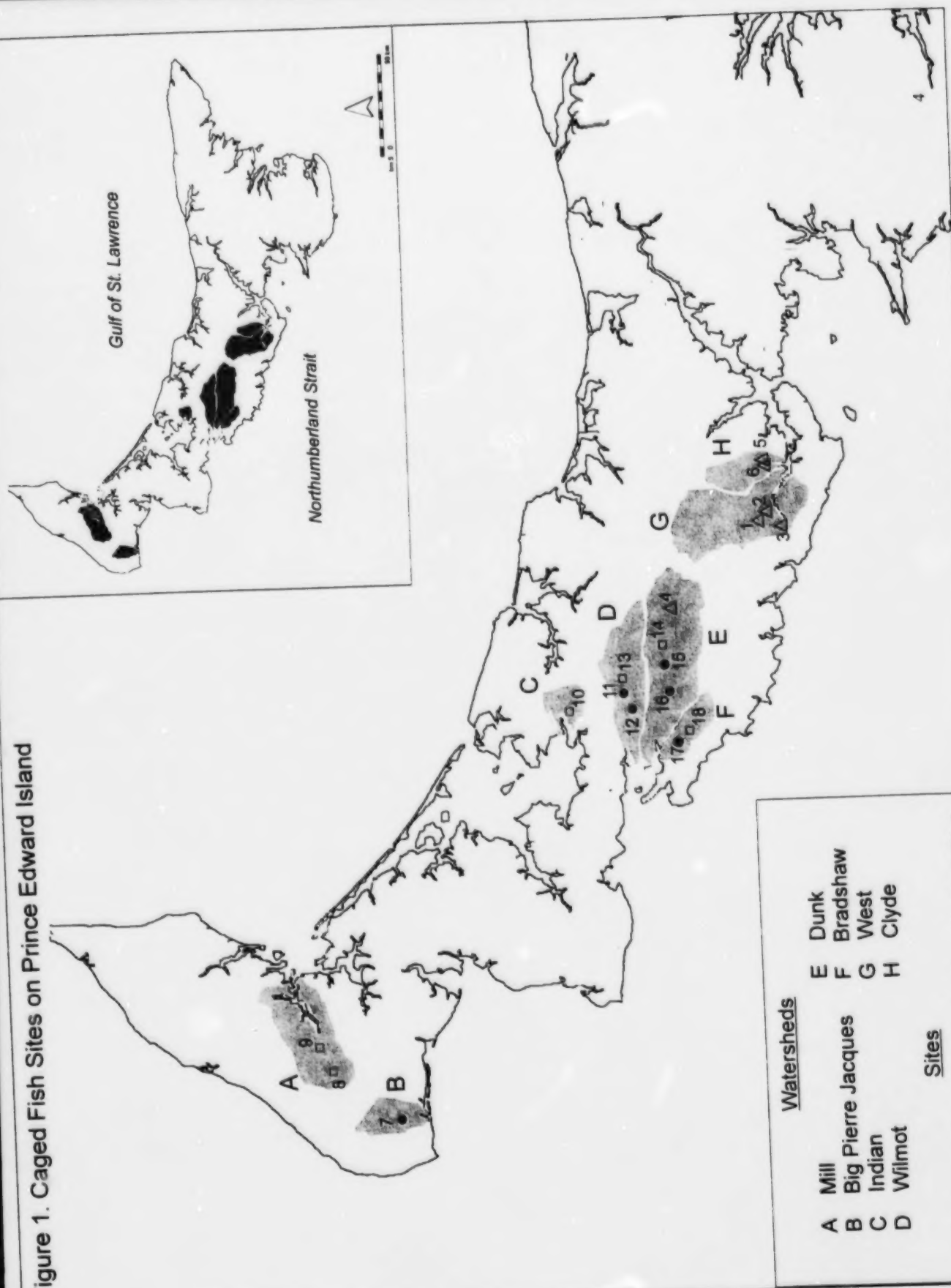


Table 1 Site numbers and their respective rivers used in the rainbow trout caging study. The sites are grouped into low, medium, and high risk groups based on the presumed potential for agricultural input (observational and hydrogeological). The sites are listed from lowest → highest estimated risk potential.

Risk Group	Site #	River
Low	6	Clyde
	5	Clyde
	3	West
	1	West
	2	West
	4	Upper Dunk
Medium	10	Indian
	8	Mill
	9	Mill
	18	Bradshaw
	14	Dunk
	13	Wilmot
High	7	Big Pierre Jacques
	16	Dunk
	11	Wilmot
	17	Bradshaw
	15	Dunk
	12	Wilmot

2.2 Caging of Rainbow Trout/Collection of Wild Brook Trout

2.2.1 Cages

The basic design of the cage is illustrated in Figure 2. The cages were constructed of vinyl-coated wire mesh and fastened with wire clips. Square pieces of burlap were attached to the top of the cages to provide camouflage for the cage itself and shading for the trout. In addition, the cages were placed beneath overhanging vegetation to avoid direct sunlight. Labels were attached to the top of the cages, identifying their purpose and providing a contact phone number.

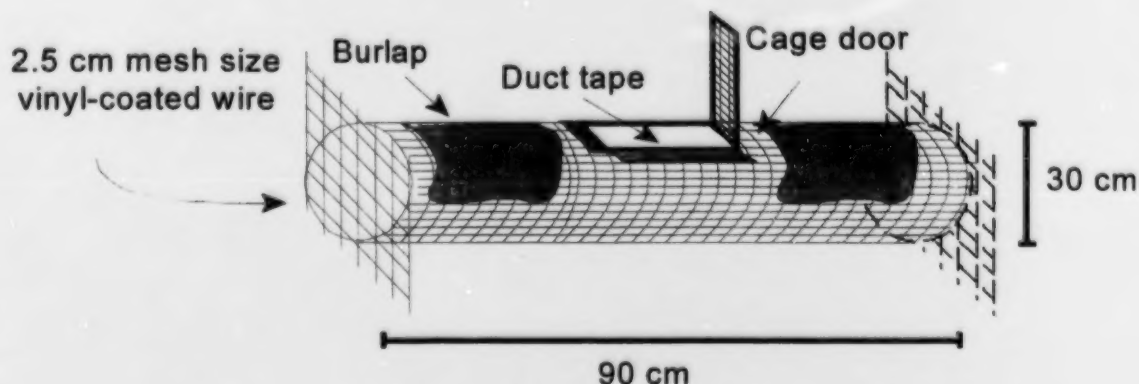


Figure 2 Design specifications of cages used to keep rainbow trout in rivers for 21-42 days.

2.2.2 Placement of Fish in Cages

Approximately 18 month old rainbow trout (*Oncorhynchus mykiss*) were purchased from the Cardigan Fish Hatchery, Cardigan, PEI.

The fish were placed in cages over three separate time periods. Table 2 indicates that caging time periods and number of exposure days for each set of cages deployed.

Table 2. Exposure periods and dates of deployment of rainbow trout held in cages

Group	Dates	# of Exposure Days
I	Week of July 13 - Week of August 3	21
II	Week of July 27 - Week of Sept 7	42
III	Week of August 24 - Week of Sept 14	21

The rainbow trout were transported to the rivers in an insulated tank equipped with a portable air compressor. The cages were weighted which also provided a more natural substrate for the fish and reduced water velocity. At each site, approximately 20 fish were placed in each cage. The door on the fish cage was then secured with nylon cable ties. Cages were placed so that the top was 10-50cm below the surface of the water. The caged fish were visited weekly and fed commercial fish food from the hatchery. In addition, any dead or damaged fish were removed and the cages were cleaned. Most mortality (1-3 fish per cage) occurred within the first week of placement in cages. In total, 990 fish were sampled throughout the caging study.

A few problems resulted in variations in the exposure times of some fish. First, in Group I, the cage at Site #1 had been tampered with, opened, and the fish removed or released, which required a replacement set of fish be deployed two weeks later. These replacements were then left in the river for 42 days, thus becoming Group II fish. Second, the fish from site #12, Group II were inadvertently removed for steroid sampling after being in the river only 1 week. Lastly, all of the fish from site #12, Group III were removed or released from the cage.

2.2.3 Collection of Wild Brook Trout and Sampling for Gonadal Histology and Circulating Sex Steroids

Wild brook trout were collected during two periods in 1998. The first collections occurred from May 21-26 and the second took place from September 11-14. Brook trout were sampled for gonadal histology in the spring and for circulating levels of gonadal sex steroids in the fall. For both sampling periods, approximately 20-30 fish were collected using a backpack electroshocker (Smith Root, Vancouver, WA, USA) from each site. For gonadal histology sampling (May), captured fish (9.8-23.3 cm; 24-130 g) were euthanized by concussion and spinal severance, gonadal tissues removed and preserved in neutral buffered formalin. For non-lethal blood sampling (September), fish (13.7-22.2 cm; 23-238 g) were anaesthetized with MS 222 (tricainemethane sulphonate), bled, revived and then released. Blood (≥ 0.5 mL) was collected using heparinized syringes then placed in heparinized tubes on ice. Plasma was isolated using centrifugation, and flash frozen in

liquid nitrogen. Sex steroid concentrations were determined at the Canadian Centre for Inland Waters (CCIW) using radio-immunoassays according to published protocols (McMaster *et al.* 1992).

2.2.4 Caged Rainbow Trout Sampling

At the completion of the exposure periods, fish were removed from cages in small groups (5-8) to avoid excessive waiting periods in a holding bucket and thus minimizing stress that is known to alter the circulating steroid levels of fish (Jardine 1994; McMaster *et al.* 1994). Individually, fish were anaesthetized in river water containing MS-222. The length and weight of each fish was measured so that condition factors could be calculated. Blood was extracted from the caudal vein using 22- or 23-gauge needles with 5 mL heparinized vacutainer blood collection tubes. In some cases, if blood collection was not sufficient using the needle, the caudal peduncle was severed and any dripping blood was collected directly into the vacutainer tube. Blood samples were stored on ice until centrifugation at the laboratory or in the portable centrifuge. Fish were weighed to the nearest 0.1 g and the fork length measured to the nearest 0.1 cm. After severing the spinal cord at the base of the brain, the gonads were removed, placed in a tissue cassette, and preserved in 10% buffered formalin.

After transportation to the laboratory, which was within 2 - 10 hours of collection, blood samples were centrifuged for 15 minutes at 1500 rpm in a refrigerated centrifuge (approx. 5°C). The plasma was transferred to 1.2 mL cryovials and flash frozen in liquid nitrogen. And subsequently stored -80°C. Some samples were handled slightly differently, due to the availability of certain equipment (Group II, sites #2, 10, 11, 13, 14, 15, 16 and Group III, sites # 1, 3, 5, 6). These samples were centrifuged in a portable centrifuge for 10 minutes at a high speed setting at room temperature within 2 - 6 hours of collection. The plasma was transferred to cryovials and frozen immediately in liquid nitrogen as above.

Once all the blood samples had been collected, they were transported to CCIW for the determination of vitellogenin levels and circulating plasma testosterone concentrations.

2.3 "Immature" vs. "Mature" Fish Identification

There were unexpectedly some mature fish (primarily males) present in the cages (about 20%). As the results of both the vitellogenin and steroid assay can be influenced by the presence of mature or maturing gonads, attempts were made to separate the fish into two classes: "mature" and "immature". If the gonads were very small and could not be visually identified as "male" or "female" in the field, the fish was considered "immature" and if the fish had gonads that were identifiable in the field, they were classified as "mature". Sex identification was later confirmed in the lab by staining the formalin-preserved gonad sections with acetocarmine and examining the gross morphology of the tissues under a light microscope. Note that only the data for the "immature" fish is presented in this report.

2.4 Plasma Assays

Vitellogenin was measured using an enzyme-linked immunosorbent assay (ELISA) (Sherry *et al.* 1999). The analytical technique used to determine concentrations of circulating steroids in the plasma was a radio-immunoassay (McMaster *et al.* 1992).

2.4.1 Caged Fish Steroid Assay

Plasma samples were prioritized based on the potential risk for agricultural input at the particular site. For each group, some samples from the lowest risk sites were analyzed for comparison with samples from the highest risk sites. In total, 327 male and female plasma samples were analyzed for testosterone. Only the results of the immature fish plasma analyses are presented in this report.

2.4.2 Wild Fish Steroid Assay

Plasma was also analyzed for steroids from the wild fish and examination of 17 β -estradiol and 11-ketotestosterone were used to determine the gender of the released fish (that could not be sexed externally). The fact that females normally produce substantially higher concentrations of estradiol than males and females do not produce 11-ketotestosterone was used for gender determinations. Testosterone concentrations were also calculated to

determine if there were differences in circulating steroid concentrations in the high intensity farming areas when compared to the reference sites.

2.5 Gonadal Histology of Wild Fish

Gonadal tissues from the wild fish were preserved in formalin and processed for histological analysis using established protocols at the Veterinary College, University of Guelph. Briefly, tissues were embedded in paraffin, sectioned using a microtome, transferred to slides, stained with hematoxylin eosin and varnished sealed. At CCIW, the sections were viewed under a microscope and the entire gonad was examined in males for the presence of testis ova. For each site, several females were also examined for comparison purposes.

2.6 Japanese Medaka Sediment Assay

2.6.1 Fish

A brood stock of Japanese medaka (*Oryzias latipes*) was originally obtained from a population maintained at Trent University and was supplemented by medaka purchased from Ward's Biological Supply (St. Catharines, ON). The fish were kept in 20 L tanks in filtered well water. The tank water was maintained at 23-26 °C with individual heaters. The fish were fed a combination of brine shrimp and flake food twice per day.

2.6.2 Egg Collection and Preparation

Eggs were collected from female medaka 1-2 hours after oviposition. Eggs were removed from females and placed in petri dishes containing rearing solution. The embryo-rearing solution consisted of 1% NaCl, 0.03% KCl, 0.04% CaCl₂, 0.163% MgSO₄, and a few drops of methylene blue in 100 mL distilled water (Kirchen and West 1976). Eggs were checked for fertilization under the dissecting microscope.

2.6.3 Sediment Collection

Sediment was collected as close as possible to the rainbow trout caging locations, usually within 1-2 metres of the cages. Composite sediment samples were collected using Teflon® core tubes that were then combined in a stainless steel bowl. After manual stirring with a stainless steel spoon to homogenize the samples, the sediment was transferred to 500 mL mason jars. The sediment was frozen (-20 °C) upon return to the lab. On the day before exposure began, individual sediment samples were removed from the freezer and transferred to a refrigerator (4 °C) to thaw slowly. On the day of the exposure, the sediment was removed from the refrigerator a few hours before use to bring it to room temperature.

2.6.4 Sediment Solution Preparation

Any water that had collected at the top of the Mason jar was decanted. Approximately 100 g of sediment was weighed into a 250 mL beaker and mixed with 100 mL of rearing solution. The mixture was manually stirred for 2 min, then filtered through four layers of new cheesecloth. The strained filtrate was then stirred with a magnetic stir bar for approximately 2 min. This filtrate was then diluted 1:5 with rearing solution to make a 20% sediment solution (10 mL sediment filtrate:40 mL rearing solution). For each sediment treatment, 1mL of the resulting solution was transferred into twenty 2 mL glass vials. The sediment solution was stirred continuously during the transfer in order to maintain the consistency of the solution.

2.6.5 Exposure

The method used was modified from a static, non-renewable exposure utilized in other studies with exposure of medaka embryos to various contaminants (Wisk and Cooper 1990; Harris *et al.* 1994; Gray and Metcalfe 1999). One egg was placed in each of the glass vials and sealed with a Teflon®-lined cap. The vials were kept in an incubator at 25±2 °C under a 16:8h light:dark photoperiod. Eggs were observed through the walls of the vial underneath a dissecting microscope every two days. After 6 days of exposure, the caps were removed briefly and the sediment solution in each vial was aerated with approximately

10 mL of ambient air using a glass pasteur pipette. On day 12 of exposure, the eggs were removed from the vials and placed in a petri dish containing rearing solution. This was done because it was observed that few eggs hatched in the vials when surrounded or partially covered by sediment by day 12 which may have been the result of larval suffocation or burial. Prior to inflation of the swim bladder, larval medaka hatching into soft sediment on the bottom were unable to swim and remain above the substrate. The embryos in the petri dishes were observed daily for hatched larvae, inflation of swim bladders, and any mortality. Successful inflation of a swim bladder and the lack of any obvious mortality were taken as an indication of a 'healthy' embryo (Marty *et al.* 1990a). Observations were ended on day 22 and any dead or unhatched embryos noted.

2.7 Statistical Procedures

The data from the medaka sediment assay were separated into the three risk categories for statistical analysis. Statistical analysis was performed using Systat® Version 6.01 and Microsoft® Excel 97. All data were normally distributed (Kolmogorov-Smirnov tests) and ANOVAs were used to detect differences between risk groups for mortality, swim bladder inflation, and unhatched embryos. Statistical analysis of circulating plasma testosterone levels in the caged fish were conducted using the Kruskal Wallis non-parametric analysis between the higher risk sites and the lowest risk site. Steroid data from wild fish was not normally distributed and was SIN transformed. ANOVA was used to compare steroid levels between sites for each sex.

3.0 RESULTS AND DISCUSSION

3.1 Vitellogenin Assay

Vitellogenin (Vg) is a large phosphoglycolipoprotein produced in the livers of non-mammalian vertebrates. Vitellogenin is released into the bloodstream and taken up by developing oocytes where it is converted into two proteins; lipovitellin and phosvitin that are the major components of the egg yolk (Wallace and Bergink, 1974; Hadley 1992). Under

normal circumstances, only egg-producing female fish will produce vitellogenin (Donaldson 1990; Wahli *et al.* 1981; Jobling *et al.* 1996). This is an estrogen-specific process and is not induced by other gonadal steroids (Hadley 1992).

The specificity of vitellogenesis makes it an ideal biomarker of exogenous exposure to estrogenic compounds. Male fish and immature fish will produce this protein when exposed to sufficient concentrations of estrogenic chemicals (Harries *et al.* 1996, 1997; Jobling *et al.* 1996; Sherry *et al.* 1999). *In vitro* and *in vivo* assays have been developed to detect exposure to environmental 'estrogens' for fish, amphibian, and turtle models (Jobling and Sumpter 1993; Pelissero *et al.* 1993; Palmer and Palmer 1995; Palmer *et al.* 1998; Sherry *et al.* 1999).

Preliminary analysis was conducted on a selected number of samples to determine whether detectable levels of vitellogenin were present in the fish plasma samples. In the first few samples of male rainbow trout plasma, the data were not consistent with normal binding of vitellogenin in the assay. The binding trend indicated non-detectable levels at low and high dilutions, while showing a significant increase in Vg concentrations at middle dilutions. After reanalyzing the same samples and a selection of samples from other sites, it was determined that the problem was likely within the plasma samples and not with procedural errors. A few of the plasma samples were then analyzed using polyacrylamide gel electrophoresis (PAGE) to determine whether Vg-like proteins were present by observing the separation of the protein bands in the plasma. This method showed the absence of apparent Vg in the samples. Western blotting methods also failed to detect protein bands that cross-reacted with the antibodies to rainbow trout Vg.

It is likely that the time from blood collection to centrifugation had been too long (e.g. ranged from approximately 2-10h); although the samples were kept on ice, the vitellogenin and other plasma proteins might have become degraded by protease activity. The aberrant results may then be explained by pieces of the large Vg protein, or protease-modified pieces of other plasma proteins, binding to the antibody, causing a false positive reading and thus obscuring the actual amount of Vg in the samples. Further analyses were

conducted on twenty-one blood samples taken from fish kept in the lab at UPEI that had been centrifuged almost immediately following blood collection. No Vg was detected in any of the lab samples from male fish and plasma from two female fish contained low levels of Vg (about 400 ng/mL) which was consistent with what one would expect to see in unexposed females of that sexual maturity (J. Sherry, pers. comm., 1998). Due to assumed degradation of the protein during sampling handling and preparation, it was decided that further analysis would not yield interpretable data and no additional samples were analysed. Attempts to find another assay that would yield comparable information regarding calcium or phosphorous levels as indirect indications of the level of Vg in the samples, were not successful. Calcium is an insensitive indicator and would not detect the anticipated levels of Vg (S. Brown, pers. comm. 1998), and phosphorous is extremely labile being rapidly lost from Vg upon storage at 4°C (J. Sherry, pers. comm. 1998).

3.2 Steroid Assays

3.2.1 Circulating Testosterone in Caged Rainbow Trout

Reductions in circulating steroid levels in fish have been associated with alterations in gonadal size, fecundity, age to maturation and expression of secondary sexual characteristics after exposure to bleached kraft mill effluents (McMaster *et al.* 1991, Munkittrick *et al.* 1991, 1992a,b). Exposure to environmental estrogens has also been associated with reduced testosterone levels in male rats (Blake and Boockfor 1997), in male alligators (Guillette *et al.* 1994) and in male carp (Folmar *et al.* 1996).

Figure 3 shows the mean testosterone concentrations for the immature rainbow trout analyzed from each caging group. The sites are listed in increasing estimated risk potential to agricultural run-off. Differences between sites were detected using a Kruskal-Wallis test.

For the male fish in Group I (21-day exposure, early in growing season), there appeared to be no pattern in testosterone levels. Variability between the two low risk sites was larger than any of the differences in the high risk sites. The large variability (even in the high risk

sites) may be due to variability in the gonadal development of these fish. No gonad weights were recorded for these fish although attempts were made to separate the visually less mature fish from those with mature or maturing gonads. Testosterone levels in the Group II (42 day, mid-season exposure) immature male fish did not show any trends; the testosterone levels were very similar, with the exception of the immature males from site 17, that showed significantly lower levels of testosterone ($P < 0.05$). For the immature males from Group III (21-day, late season), sites 7 and 17 showed significantly higher testosterone levels than the lowest risk site but variability in the high risk sites was greater than the difference between high and low risk sites and Site 6 blood samples were processed differently, making comparisons weak.

For the Group I immature females, it appeared that the concentration of testosterone increased as the estimated risk increased however only those fish from the highest risk site (Site #12) had significantly higher testosterone when compared to the lowest risk site (Site #6). There were no significant differences in testosterone levels observed among the immature female fish from Group II. In Group III, it appears as though all immature females had significantly different testosterone concentrations than those females held at the lowest risk site (Site #6). The steroid hormone levels at the first three sites in ascending risk were significantly higher, while the highest risk site (Site #15) was significantly lower. This variability within the high risk sites however suggests that no valid comparisons can be made as the blood samples from the low risk site were processed differently which could alter the interpretation of the data.

One major factor contributing to the lack of significant trends observed in the steroid levels was the high variability of the data. Field studies have shown that effects of capture and handling stress can change reproductive steroid levels with small changes in sampling procedures (Jardine 1994; McMaster *et al.* 1994). Other studies have shown that changes in steroid levels in a sample are due to length of holding time prior to centrifugation (S. Brown, pers. comm. 1998). Large variability in gonadal development of fish within and between sites could also account for the variability in the steroid levels between sites.

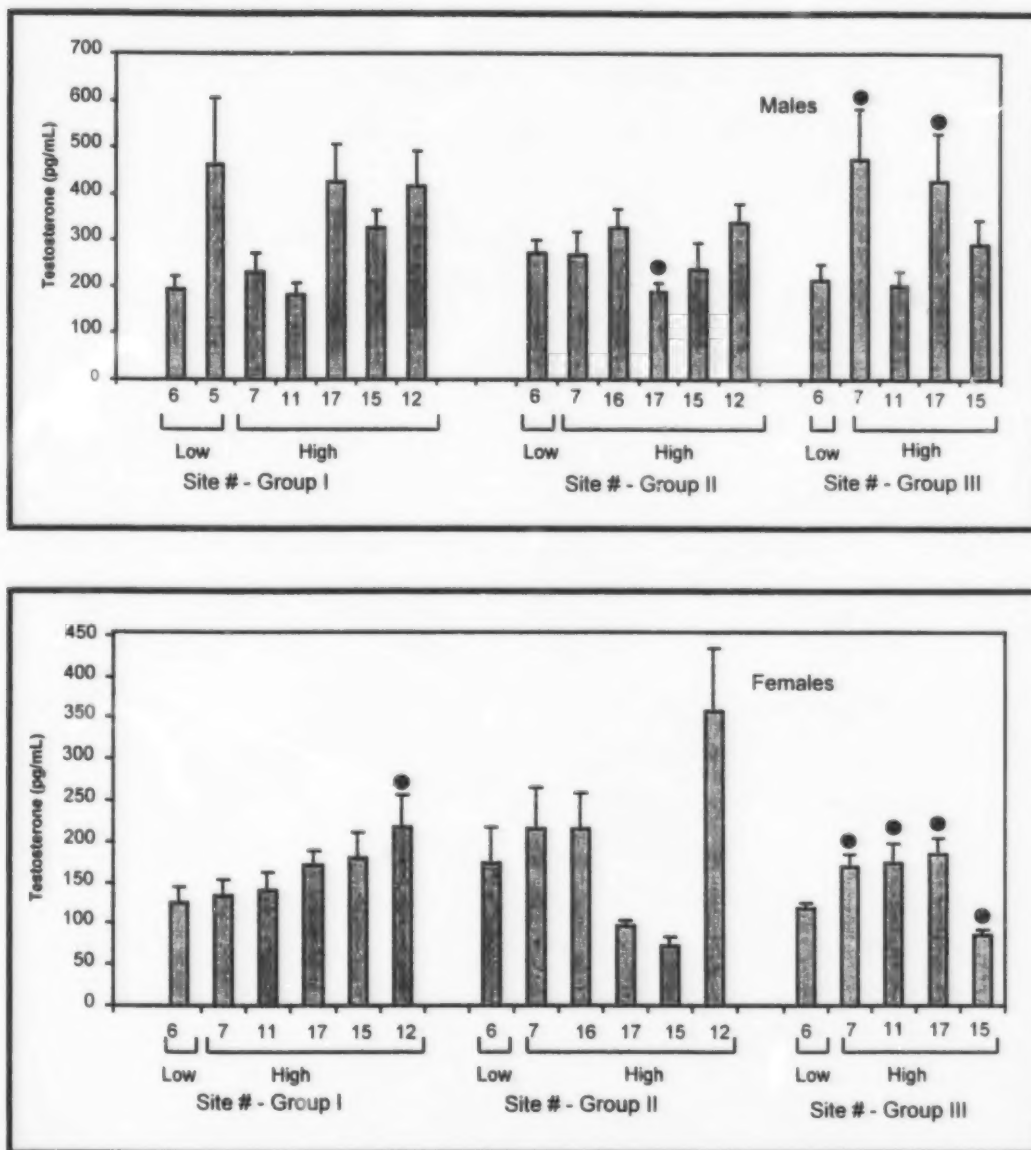


Figure 3: Mean testosterone concentrations ($\pm$ SE) in male and female rainbow trout from the three exposure periods (Groups I, II & III). Sites are listed in increasing risk to presumed agricultural run-off exposure. ● indicates a significant difference from the lowest risk site (Site 6) ($p < 0.05$).

Acknowledging that the assigned risk ranking was somewhat arbitrary, there were no consistent trends between the levels of testosterone in either immature male or immature female fish and the assumed risk of exposure to pesticides. A depression in steroid hormones is often observed with fish exposed to pulp mill effluents (McMaster *et al.* 1991; Munkittrick *et al.* 1991, 1992a,b; Van Der Kraak *et al.* 1992). In many such studies, the majority of fish being sampled are mature fish. Adult fish possess negative feedback mechanisms to compensate for the exposure to exogenous hormonal agents i.e. the system will stop producing the respective hormones in response to EDC exposures. Conversely, it is suspected that juvenile fish may possess only positive feedback mechanisms (K. Munkittrick pers. comm. 1998). Juvenile fish exposed to sediment near a steel mill, pulp mill effluent, and sewage effluents have displayed an increase in steroid hormones (K. Munkittrick, pers. comm. 1998). The positive feedback response to exogenous hormones or hormonal stimulation, in these cases, may be to stimulate production of endogenous hormones. Although speculative, such a mechanism would explain the increases in steroid hormones seen in some of the caged fish at higher risk sites.

3.2.2 Circulating Steroids and Gonadal Histology in wild brook trout

Gonadal histological analyses of wild brook trout collected in May 1998 showed no incidence of testis-ova in the fish from any of the sites samples. Lack of testis-ova in fish indicates that sufficient estrogenic chemicals associated with this condition were not present or that the specific timing of such an exposure was not appropriate for the development of this condition.

Concentrations of circulating 11-ketotestosterone in males and estradiol in females was used to determine specific gender of the wild fish. Once sexed, 11-ketotestosterone and testosterone in males and 17 β -estradiol and testosterone in the females were compared between the sites. No differences between any of the sites ($P > 0.05$ ANOVA) was found for either sex, with any of the steroids (Figure 4). The absence of measurable effects on steroids indicates that agricultural activities were not influencing circulating levels in fish collected at that time. Since samples were collected during the latter periods of fungicide

applications and top kill applications, it is possible that effects of chemicals applied earlier in the growing season would not be measured.

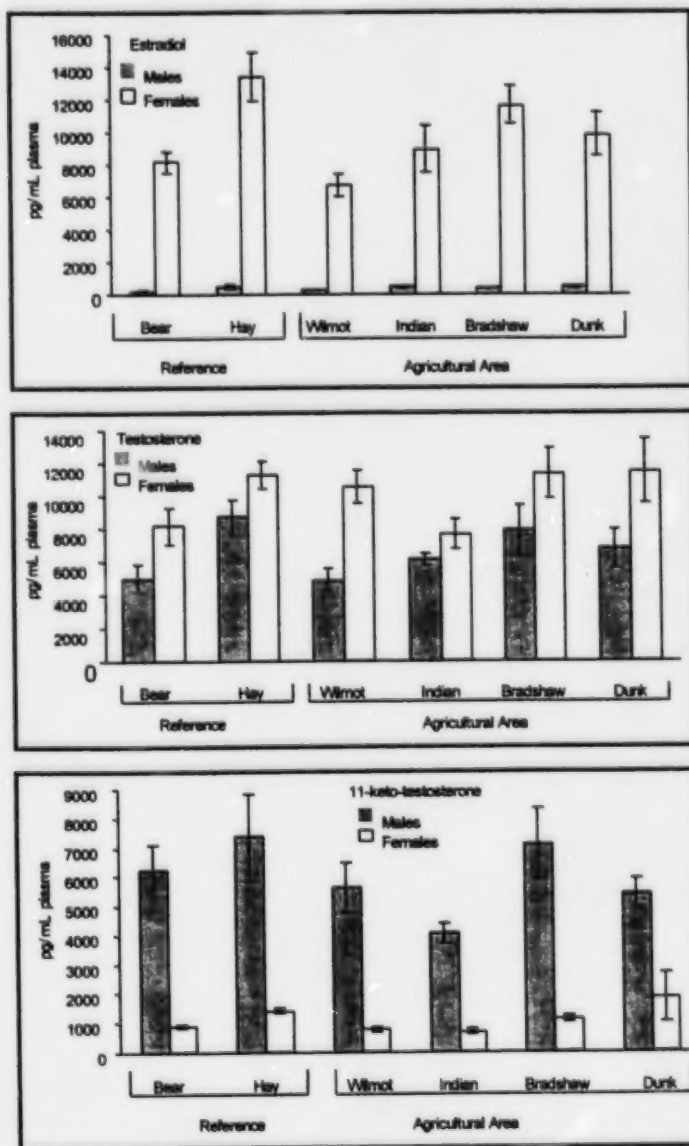


Figure 4 Circulating steroid concentrations in wild brook trout plasma

3.3 Rainfall Data

In order to determine whether there was a correlation between high rainfall events, potentially causing high runoff from fields, and testosterone levels in groups of fish, daily rainfall data was obtained from Environment Canada's Atlantic Climate Centre for various climate stations located near the caging study sites. The locations of the climate stations are indicated on Figure 5. The Elmwood station was used for the low risk sites as there were no stations located closer to that particular region. Similarly, stations nearest the other sites were used to indicate approximate rainfall events in those regions.

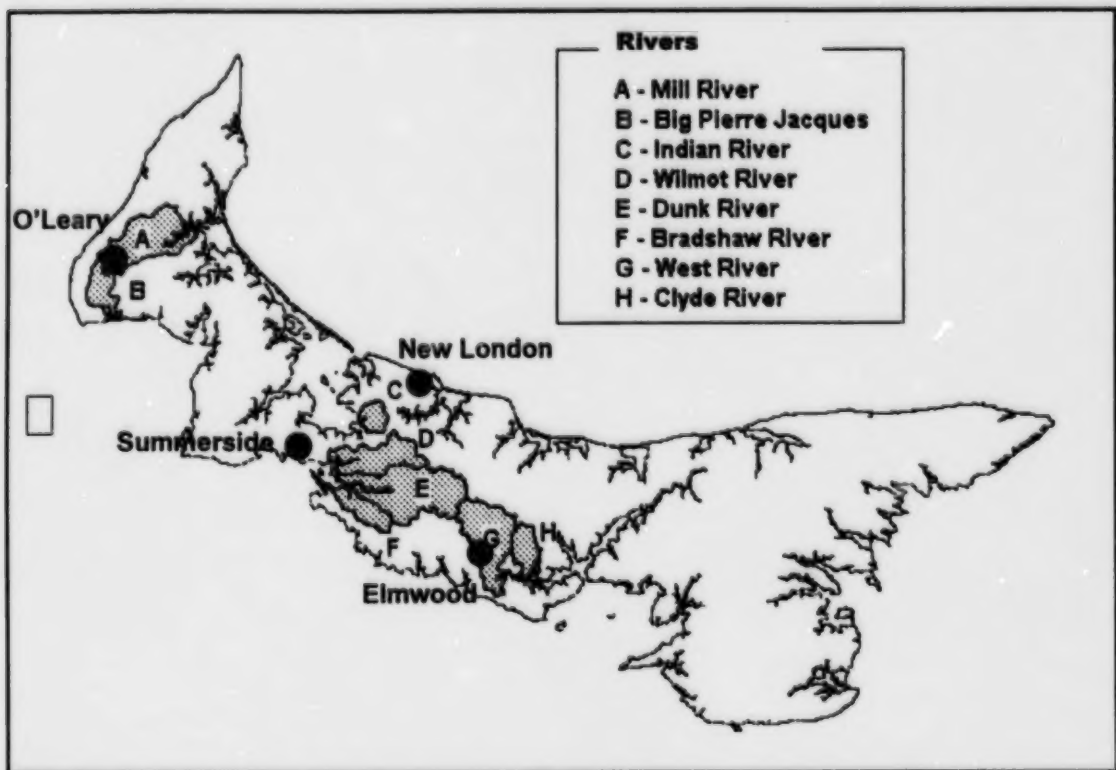


Figure 5 Map of Prince Edward Island indicating Environment Canada's climate stations used for rainfall data (●).

Total rainfall amounts were compared with testosterone concentrations in fish; if runoff was a contributing factor to the changes in steroid concentrations observed in the fish, it was hypothesized that potential EDC inputs might be correlated with rainfall amounts. Using regression analysis, total rainfall at the closest climate station to each caging site was tested against mean plasma testosterone concentrations (males and females tested separately) and against mean condition factors (sexes combined). There was no significant relationship between rainfall amounts and either testosterone levels or condition ($P > 0.05$). What could not be determined from the available data, however, is whether site specific differences may cause runoff under varying conditions.

3.4 Fish Condition Factors

A condition factor ($\text{weight} / (\text{length})^3 \times 100$) was used to assess the relative health of the caged fish. Figure 6 displays the mean condition factors for all fish in the three risk categories.

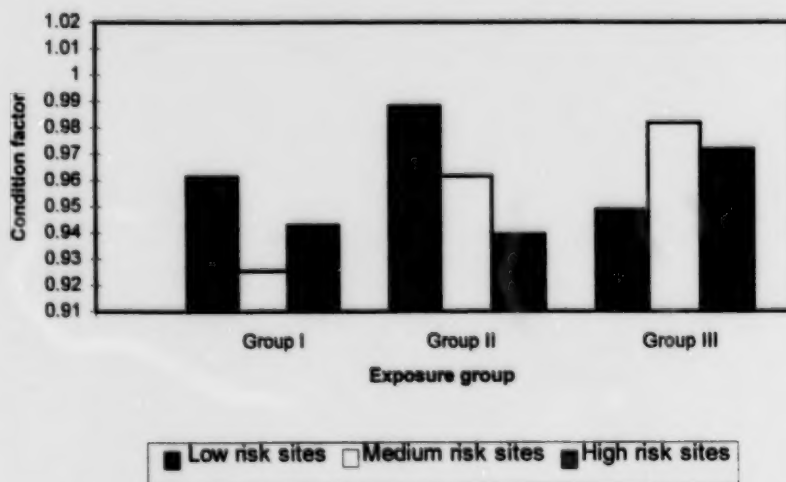


Figure 6: Mean fish condition factors for each exposure group in all three risk categories

In Group I, the medium risk group had lower average condition factors than those fish caged at the lower or higher risk sites. For the fish in Group II, the average condition factors decreased as the presumed risk of agricultural run-off increased. In Group III, it was the fish held at the lowest risk sites that had the lowest average condition factor.

The differences in fish condition may have been caused by differences in food availability, at each site, as well as current flow in each river. The latter could affect the condition factor by influencing the amount of energy expended by the fish, affecting weight gain and/or loss. As the current flow for each river was not measured during this study, it is not possible to assess the degree to which the condition factors were affected by the various flow rates.

As the results from Group III indicated that the fish situated at lower risk sites had a lower condition factor relative to fish located at medium and higher risk sites, there does not appear to be a consistent relationship to associate reduced condition with the potential for increased agricultural contaminant exposure.

3.5 Japanese Medaka Assay

Exposure to chemicals and changing environmental conditions generally have the most impact during critical early life stages of development and differentiation (McKim 1977; von Westernhagen 1988). Significant fish mortality or developmental impact at these early life stages can have a considerable effect on the recruitment of the fish population. The embryolarval toxicity test assesses acute endpoints related to exposure of fish to developmental stressors.

There were certain abnormalities observed in the medaka embryos exposed to sediment from all sites in all risk groups such as: varying degrees of partial hatch (only the head or the yolk sac protruding), early liberation from the chorion (day 5), some cranial/facial and spinal deformities, hatching with only one pectoral fin and a general developmental lag observed in some embryos although this but could not be properly assessed due to an inability to see some embryos clearly enough depending on the type of surrounding sediment. Although these problems were more often observed in the higher risk treatments, no significant differences between any of the three groups were detected using the Chi-square test.

Figure 7 summarizes the medaka assay results for mortality and hatching success/rates. Mean mortality increased with estimated risk of exposure potential to agricultural run-off and was significantly higher in medaka embryos exposed to sediment collected from high risk areas compared with the low risk areas (ANOVA; $P < 0.05$). Additionally, the mean number of embryos that successfully hatched and inflated a swim bladder decreased with the estimated risk of exposure potential to agricultural run-off and was significantly higher in the low risk group compared to the high risk group ($P < 0.05$). The mean number of unhatched eggs remaining at the end of the exposure period was not correlated with estimated risk of exposure and was not significantly different between the low, medium, and high risk groups ($P > 0.05$).

The mean time to hatch for medaka was also assessed for the three risk groups. Of the embryos that hatched, the eggs exposed to sediment from the medium and high risk sites took significantly longer to hatch than eggs exposed to sediment from low risk sites (ANOVA; $P < 0.05$) (Figure 8).

Again, it is acknowledged that the classification of individual sites into risk categories was based on the surrounding land use (i.e. cleared, agricultural land versus uncleared or forested land) and hydrogeological data as well as some semi-quantitative observations of the sites. The risk ranking employed therefore may not be a true reflection of actual risk.

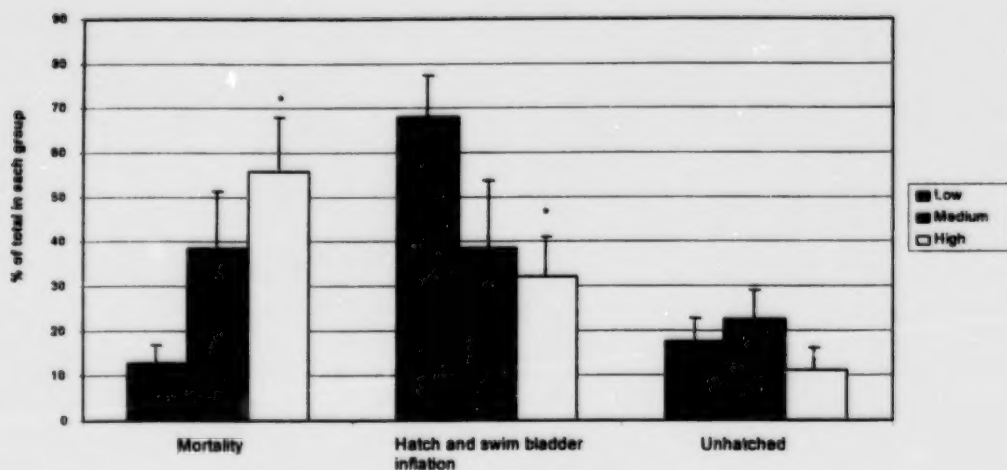


Figure 7. Mean percent mortality, hatch & successful swim bladder inflation and unhatched larvae ($\pm$ SE) for medaka embryos exposed to sediments collected from rainbow trout caging sites grouped into low, medium and high risk classes. The * indicates a significant difference from the lowest risk group ($p < 0.05$).

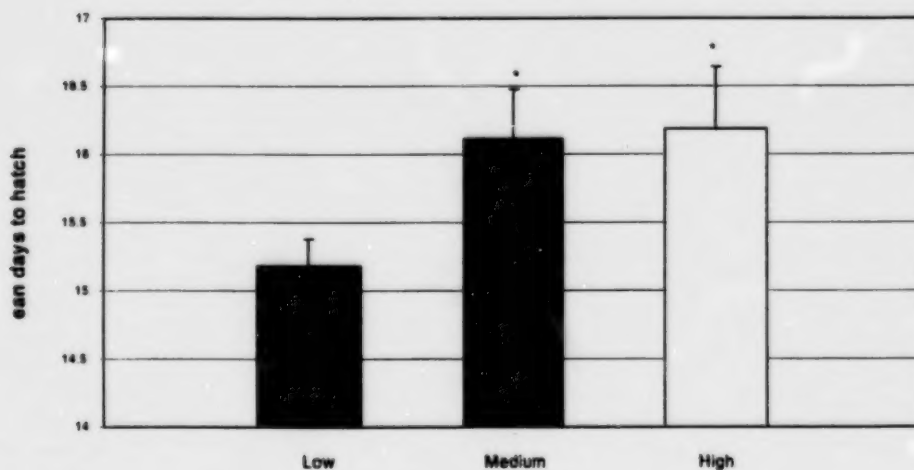


Figure 8. Mean days to hatch ($\pm$ SE) for medaka embryos exposed to sediments collected from rainbow trout caging sites grouped into low, medium and high risk classes. The * indicates a significant difference from the lowest risk group ($p < 0.05$).

The earlier mean hatching time for the embryos from low risk groups could be due to a number of causes. The hatching process in fish is mediated by the release of a hatching

enzyme (chorionase) in the cephalic region of the embryo (von Westernhagen 1988). Impacts on incubation time can be due to the relative development of the embryos or due to impacts on the hatching enzymes. For example, temperature increases developmental rates and may result in well-developed embryos hatching early. As well, changes in levels of the hatching enzymes may result in either embryos hatching that are underdeveloped and cannot survive (i.e. hatching enzymes stimulated) or embryos which are ready to hatch but are unable to liberate themselves from the chorion (i.e. hatching enzymes inhibited). Common in sublethal toxicity tests with fish embryos is the unequal distribution of hatching enzyme resulting in partial hatch (von Westernhagen 1988). This most often occurs in the cephalic region, but in this study, when there was hatching failure, tails, yolk sacs, and heads were all observed to be protruding with the embryos apparently unable to free themselves entirely.

Environmental pollutants have varying effects on hatching time (von Westernhagen 1988). Oil products have reduced the hatching times in medaka embryos (Leung and Bulkley, 1979), while metals such as zinc, cadmium, copper, and mercury may prolong the incubation period in fish (von Westernhagen 1988). Two common fungicides used on PEI, Acrobat-MZ and Tattoo-C, do not affect medaka embryo hatching time (Teather *et al.* 1999).

Water quality parameters such as pH and ambient oxygen levels also effect hatching time. Hatching may be delayed or prevented entirely at pH levels below 5.5 (von Westernhagen 1988). While pH levels of the sediment were not measured in this study, the rearing solution was buffered and would be expected to ameliorate at least part of the effect of the pH.

Development can be delayed and incubation times lengthened if low oxygen levels are sustained throughout the incubation period (von Westernhagen 1988). Yamagami *et al.* (1983) noted that for medaka embryos kept in highly oxygenated medium, the secretion of hatching enzyme was retarded. Conversely, if oxygen levels are decreased after a certain stage of development, hatching is actually stimulated by increased respiratory activity in

medaka (Yamagami 1970). Thus, either too little or too much oxygen may cause alterations in hatching times. Marty *et al.* (1990b) studied the effects of oxygen consumption and ammonia production on hatching in medaka embryos. They concluded that oxygen became a limiting factor, not ammonia production, when there was no air/water interface. Although the embryos in this study were kept in sealed containers, there was an air/water interface. With aeration after 6d and complete removal of the embryos after 12d it is unlikely that the embryos were exposed to anoxic conditions however measurements were not made of the oxygen concentrations in the test solutions.

None of the observations of decreased health in the medaka embryos from higher risk treatment groups can be positively attributed to endocrine disruption. However, there are many developmental and hatching mechanisms that are dependent on proper endocrine functions that may have been affected directly or indirectly by exposure to contaminated sediment.

Overall, the embryos exposed to sediment collected from the low risk sites were healthier than the embryos exposed to sediment collected from medium and high risk sites as measured by mortality, rates of swim bladder inflation, and mean time to hatch.

4.0 Conclusions and Recommendations

In conclusion, the vitellogenin assay was unable to accurately detect the presence or absence of vitellogenin in the plasma and was likely due to sample handling problems which should be corrected if follow-up studies are conducted. The testosterone assay in the caged fish did demonstrate significant differences in testosterone concentrations in rainbow trout held at higher risk sites when compared to the lower risk sites however there were no clear trends between assumed risk of pesticide exposure and the steroid concentrations. Clearer trends may be observable if more quantitative methods of assessing exposure, such as pesticide use patterns within the watershed or pesticide measurements within the streams, were conducted. Circulating steroids and gonadal histology in wild brook trout did not indicate differences between any of the sites, suggesting that exposure to chemicals with the potential to affect these parameters is not occurring. There was increased mortality, increased failure to inflate swim bladders and increased hatching time in Japanese medaka embryos exposed to sediment sampled from areas with increasing risk to agricultural runoff.

While not conclusive, this preliminary study does provide some evidence to support further study to verify whether agricultural activities are producing endocrine disrupting effects and, if necessary, determine the causative agents of such effects.

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